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"*Bacteroides goldsteinii* sp. nov." Isolated from Clinical Specimens of Human Intestinal Origin

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Phenotypic and phylogenetic studies were performed on an unknown gram-negative, strictly anaerobic, rod-shaped bacterium isolated from human clinical specimens. This organism was indole negative, resistant to 20% bile, produced acetic and a lesser amount of succinic acids as the major end products of glucose metabolism, and possessed a G+C content of approximately 43 mol%. Comparative 16S rRNA gene sequencing demonstrated that the unidentified bacterium was a member of the *Cytophaga-Flavobacter-Bacteroides* phylum of gram-negative bacteria and formed a close association (with an average sequence similarity of 93.6%) with the second subcluster of the *Porphyromonas* cluster in the *Bacteroides* subgroup. Phylogenetically and phenotypically it resembled *Bacteroides merdae*; however, a 16S rRNA gene sequence divergence of approximately 5.5% between the unknown bacterium and *B. merdae*, as well as distinguishable biochemical characteristics, demonstrate that the unknown bacterium is genotypically and phenotypically distinct and represents a previously unknown subline within the *Porphyromonas* phylogenetic cluster. Furthermore, a DNA-DNA reassociation value of 17.8% between isolates WAL 12034^T (the type strain of this novel taxon) and ATCC 43184^T (*B. merdae* type strain) also documented the separateness of the unknown species and *B. merdae*. Based on the phenotypic and phylogenetic findings, a new species, "*Bacteroides goldsteinii* sp. nov," is proposed. The G+C content of the DNA is 43 mol% for *Bacteroides*. The type strain of "*B. goldsteinii*" is WAL 12034^T (= CCUG 48944^T = ATCC BAA-1180^T).

The taxonomy of *Bacteroides* has undergone significant changes in the past few years (8). Studies have shown that the genus *Bacteroides* contained species representing several genera. A majority of the species previously included in the genus *Bacteroides* have been placed in the genera *Porphyromonas*, *Prevotella*, and *Bacteroides* sensu stricto (9–11). Several other genera have subsequently been described for *Bacteroides* species which do not conform to these three major groups (e.g., *Anaerorhabdus*, *Dichelobacter*, *Dialister*, *Fibrobacter*, *Megamonas*, *Mitsuokella*, *Rikenella*, *Sebaldella*, *Tannerella*, *Tissierella*, and *Alistipes*). The taxonomic positions of some other species still included in the genus, such as *Bacteroides distasonis* and *Bacteroides merdae*, remain uncertain; all of these species will ultimately be transferred to other genera (6). Furthermore, several clinically important species still await formal description.

In this paper, we report on the characterization of a group of isolates which were recovered from clinical specimens of human intestinal origin. Phenotypically, the unknown bacterium is very much like *B. merdae*; these strains were misidentified as *B. merdae* previously. However, 16S rRNA sequencing reveals approximately 5.5% sequence divergence between the novel species and its phylogenetically closest species, *B. merdae*. A DNA-DNA hybridization study also confirmed that this unknown organism was indeed distinct from its nearest valid species, *B. merdae*. Based on the phenotypic and phylogenetic findings presented here, a new species, "*Bacteroides goldsteinii*," is proposed. In addition, we describe the phenotypic tests useful in distinguishing between this novel organism and its related taxa.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The study included seven *B. merdae*-like strains, seven strains each of *B. merdae*, *B. distasonis*, and *Tannerella forsythensis*, and three strains each of *Bacteroides fragilis*, *Bacteroides vulgatus*, *Bacteroides caccae*, *Prevotella nigrescens*, *Prevotella disiens*, *Prevotella corporis*, *Prevotella intermedia*, *Porphyromonas asaccharolytica*, *Porphyromonas endodontalis*, and *Porphyromonas gingivalis* (Table 1). The novel isolates were recovered from clinical sources, such as peritoneal fluid, appendix tissue, and intra-abdominal abscess; therefore, they are likely of intestinal origin. All the clinical isolates of each species were identified by 16S DNA sequencing in our laboratory. All the strains were cultivated on Brucella blood agar (Difco, Detroit, Mich.) supplemented with 5% sheep blood and incubated anaerobically at 37°C under an N_2 (86%), H_2 (7%), and CO_2 (7%) gas phase.

Biochemical characterization. The strains were characterized biochemically by using a combination of conventional tests as described previously in the Wadsworth manual (3), plus the API ZYM and rapid ID 32A systems (API bioMérieux, Marcy l'Etoile, France), and the RapID ANA II (Remel, Inc., Lenexa, KS) system according to the respective manufacturer's instructions. All biochemical tests were performed in duplicate. Fermentation tests were performed using prereduced, anaerobically sterilized peptone-yeast-sugar broth tubes (Anaerobe Systems, Morgan Hill, CA). The strains were grown in peptone-yeast broth and peptone-yeast-glucose broth (Anaerobe Systems, Morgan Hill, CA) for metabolic end product (short-chain volatile and nonvolatile fatty acids) analysis by gas liquid chromatography (3). The MICs of seven antimicrobial agents, which were selected either as representative of a class of compound or as drugs for which MICs for quality control strains were published, were determined by the National Committee for Clinical Laboratory Standards reference agar dilution

TABLE 1. List of strains used in this study

Species	Strain (n^a)	Source(s)[b]
Bacteroides spp.		
"*B. goldsteinii*"	CCUG 48944^T (1)	Abdominal fluid
	Clinical isolates (6)	Peritoneal fluid, appendix tissue, intra-abdominal abscess
B. merdae	ATCC 43184^T (1)	Feces
	Clinical isolates (6)	Appendix tissue
B. distasonis	ATCC 8503^T (1)	
	Clinical isolates (6)	Intra-abdominal abscess
B. fragilis	ATCC 25285^T (1)	Appendix abscess
	Clinical isolates (2)	Peritoneal fluid
B. vulgatus	ATCC 8482^T (1)	
	Clinical isolates (2)	Intra-abdominal abscess
B. caccae	ATCC 43185^T (1)	Feces
	Clinical isolates (2)	Appendiceal abscess
Tannerella forsythensis	ATCC 43037^T (1)	Periodontal pocket
	Clinical isolates (2)	Periodontal pocket
Prevotella spp.		
P. nigrescens	ATCC 33563^T (1)	Gingival sulcus
	Clinical isolates (2)	Neck abscess
P. disiens	ATCC 29426^T (1)	Bartholin abscess
	Clinical isolates (2)	Bone tissue
P. corporis	ATCC 33547^T (1)	Cervical swab
	Clinical isolates (2)	Foot ulcer drainage
P. intermedia	ATCC 25611^T (1)	Empyema
	Clinical isolates (2)	Pus from human jaw lesion
Porphyromonas spp.		
P. asaccharolytica	ATCC 25260^T (1)	Empyema
	Clinical isolates (2)	Rectal abscess
P. endodontalis	ATCC 35406^T (1)	Root canal
	Clinical isolates (2)	Perirectal abscess
P. gingivalis	ATCC 33277^T (1)	Gingival sulcus
	Clinical isolates (2)	Peritoneal fluid

[a] No. of isolates.
[b] Human origin.

method (5). β-Lactamase production was tested using nitrocefin disks (Cefinase; BBL, Becton, Dickinson and Company, Sparks, MD) inside the anaerobic chamber.

Cellular fatty acid composition. Long-chain cellular fatty acids were detected with a Hewlett-Packard 5890 series II gas chromatograph, and cellular fatty acid profiles were determined by the Microbial Identification System software (MIDI, Newark, N.J.) as described previously (13). The corresponding library (ANAEROBE, version 5.0) was used in successive analyses. Peaks were automatically integrated, fatty acids were identified by equivalent chain length, and percentages of the total peak area were calculated. External calibration was done by using MIDI calibration mixture I.

DNA base composition. The mol% G+C content of DNA was determined by high-performance liquid chromatography according to the method of Mesbah et al. (4) except that the methanol content of the chromatographic buffer was decreased to 8% and the temperature was increased to 37°C.

16S rRNA sequencing and phylogenetic analysis. The 16S rRNA genes were amplified by PCR using universal primers 8UA (positions 8 to 28, *Escherichia coli* numbering) and 1485B (positions 1485 to 1507) as described previously (12). The amplified product was purified by using a QIAamp PCR purification kit (QIAGEN, Inc., Chatsworth, CA) and directly sequenced with a Biotech Diagnostic (Biotech Diagnostic, CA) BigDye sequencing kit on an ABI 377 sequencer (Applied Biosystems, Foster City, CA). The closest known relatives of the new isolates were determined by performing database searches using the BLAST software (1). Almost the full lengths of the 16S rRNA gene sequences (>1,400 nucleotides) of the unidentified bacteria and of closely related bacteria were aligned using CLUSTAL-W (http://genome.kribb.re.kr). A phylogenetic tree was reconstructed using DNA analysis software PAUP* 4.0 (Sinauer Associates, Inc., Sunderland, MA). The stability of the groupings was estimated by bootstrap analysis (1,000 replications) using the same program.

DNA-DNA reassociation. DNA-DNA reassociation experiments were carried out according to the spectrophotometric method of De Ley et al. (1a), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermal programmer.

Nucleotide sequence accession number. The 16S rRNA sequence of strain WAL 12034^T has been deposited in GenBank under accession number AY 974070.

RESULTS

The novel bacterium recovered from clinical infections of human intestinal origin was always isolated together with other anaerobes (mostly other species of the *B. fragilis* group) and/or aerobes. Four strains were isolated from appendix tissue of four patients with appendicitis, two were isolated from peritoneal fluid, and one was isolated from abdominal abscess. Moderate to heavy growth was obtained for most of the isolates on primary isolation blood agar plates. Gram staining showed that they were gram negative and rod shaped, and typical cells were 0.9 to 1.5 μm by 1.2 to 10 μm, observed by an optical microscope. Colonies on Brucella blood agar plates at 48 h were gray, circular, convex, entire, and opaque and attained a diameter of 1 to 2 mm. All of the isolates grew well (resistant to 20% bile) and blackened the *Bacteroides* bile esculin agar by hydrolyzing esculin. They all grew well anaerobically, but no growth occurred following subculture in air or in atmospheres of 2% or 6% O_2. All of the strains were resistant to the

kanamycin (1,000 μg), colistin sulfate (10 μg), and vancomycin (5 μg) special-potency identification disks. They were indole, urease, and nitrate negative. They all produced acid from cellobiose, glucose, rhamnose, sucrose, trehalose, and xylose but did not produce acid from arabinose and xylan. Using the API ZYM, rapid ID 32A, and RapID ANA II systems, all isolates of the same group produced the same profile. They were all identified as *B. merdae* with a low percentage of similarity. Positive reactions were obtained for α-glucosidase, α-galactosidase, β-galactosidase, β-*N*-acetyl-glucosaminidase, naphthol-AS-Bl-phosphohydrolase, acid phosphatase, alkaline phosphatase, leucine arylamidase, *p*-nitrophenylphosphatase, arginine arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, leucyl glycine aminopeptidase, glycine aminopeptidase, phenylalanine aminopeptidase, arginine aminopeptidase, and serine aminopeptidase. All the other tests were negative; α-fucosidase was negative in all three systems. Mannose and raffinose were fermented when tested by the rapid ID 32A system. Using Rosco diagnostic tablets (Rosco, Taastrup, Denmark), β-xylosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase (*o*-nitrophenol-β-D-galactopyranoside), β-*N*-acetyl-glucosaminidase, alkaline phosphatase, and α-arabinosidase were detected. Results for the production of β-glucosidase and α-arabinosidase differed between the different biochemical kits and the Rosco tablets. Using Rosco tablets, α-arabinosidase was detected, but with the biochemical kits it was not detected. β-Glucosidase was detected by rapid ID 32A and Rosco tablets but not by the API ZYM and RapID ANA II systems. In peptone yeast broth and peptone yeast glucose broth, major amounts of acetic acid and succinic acid and minor amounts of propionic acid, isovaleric acid, and formic acid were produced by all isolates. Long-chain cellular fatty acids analysis using Microbial Identification System software and the corresponding library provided a misidentification as *Tannerella forsythus* with a low similarity index. The principal long-chain cellular fatty acids of the isolates were anteiso-15:0 and iso-3-OH-17:0 (25 to 28% and 18 to 23% of the total, respectively). Significant amounts of 18:1 *w*9*c* and anteiso-17:0 3OH (11 to 16% and 9 to 15% of the total, respectively) were also present. Agar dilution tests showed all of the strains were susceptible to metronidazole (MIC, ≤2 μg/ml) and ertapenem (MIC, ≤1 μg/ml). Some resistance was seen with clindamycin

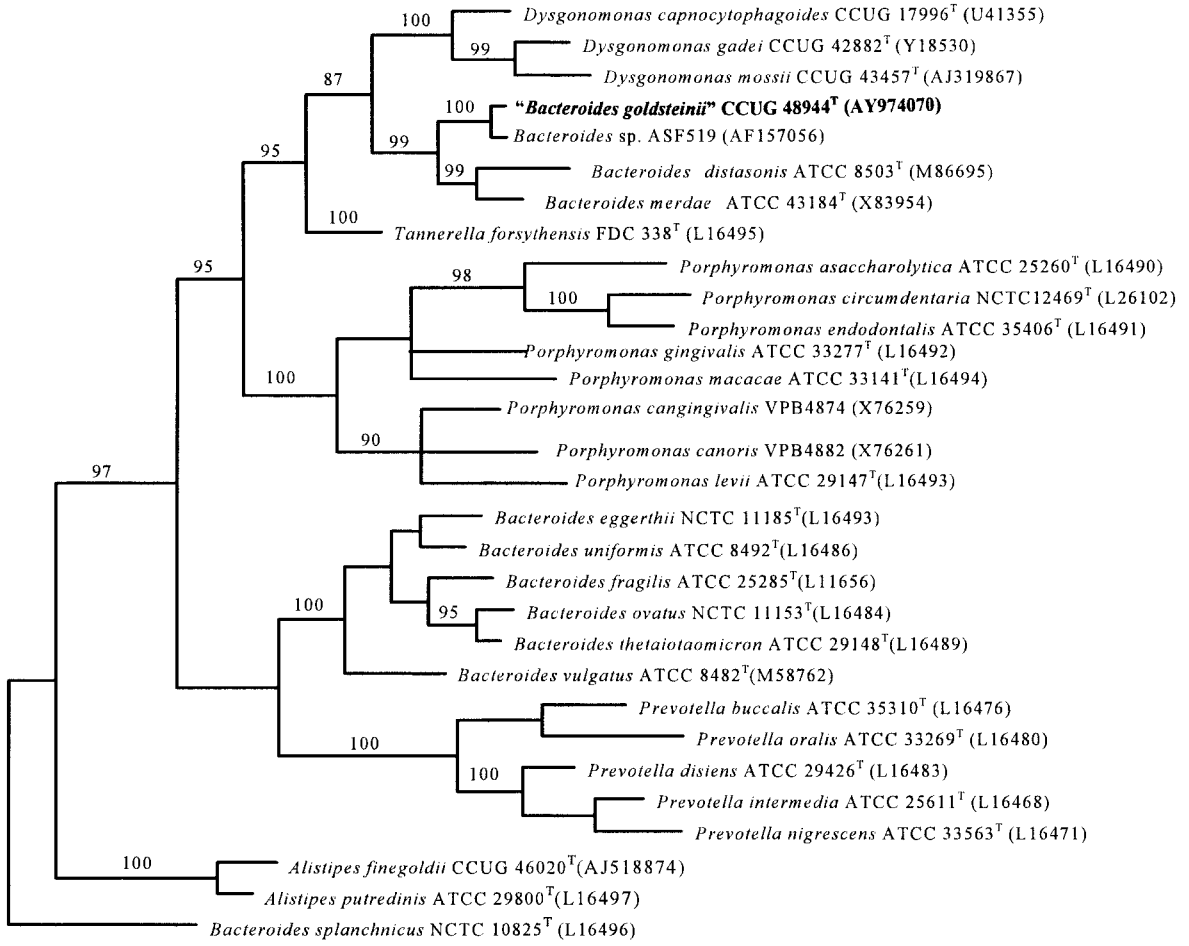

FIG. 1. Unrooted tree showing the phylogenetic position of "*Bacteroides goldsteinii* sp. nov" within the *Porphyromonas* phylogenetic cluster. The tree, constructed using the neighbor-joining method, was based on a comparison of approximately 1,400 nucleotides. Bootstrap values, each expressed as a percentage of 1,000 replications, are given at branching points. Scale bar = 1% sequence divergence.

TABLE 2. Some properties by which "*Bacteroides goldsteinii* sp. nov." can be differentiated from related taxa[a]

Characteristic	"*B. goldsteinii*"	*B. merdae*	*B. distasonis*	*Tannerella forsythus*	*Porphyromonas*	*Prevotella*	*Bacteroides* sensu stricto[c]	*Dysgonomonas*
Growth in bile	+	+	+	−	−	−	+	+
Catalase produced	v	−	+	v	v	v	v	+
Enzyme activity[b]								
β-Glucuronidase	+	+	−	+	v	v	v	v
α-Glucosidase	+	−						
β-Glucosidase	+	− or +w	+	−	v	v	v	v
Pigment produced	−	−	−	−	+	+	−	−
Major end products	A, S	A, S	A, S	A, B, IV, P, PA	A, B, IV, P, PA	A, S	A, S	P, L, S
Ratio of anteiso-$C_{15:0}$ to iso-$C_{15:0}$	5.9–10.3	4.0–5.4	4.1	22.8–95.2	<1	1.2–11.3	2.1–5.4	ND
G+C content (mol%)	43	43–46	43–45	44–48	40–55	40–60	40–48	38

[a] Data from the present study, Sakamoto et al.'s study (7), and Hofstad et al.'s study (2). Symbols and abbreviations: +, positive; +w, weak positive; −, negative; v, variable; ND, not determined; A, acetic acid; S, succinic acid; B, butyric acid; IV, isovaleric acid; P, proprionic acid; PA, phenylacetic acid; L, lactic acid.

[b] Tested by Rosco tablets.

[c] The key tests to distinguish the novel species from other species in *Bacteroides* sensu stricto are as follows: it can be differentiated from *B. stercoris, B. uniformis, B. thetaiotaomicron, B. ovatus, B. nordii*, and *B. salyersiae* by being indole negative; from *B. fragilis* and *B. vulgatus* by being trehalose positive; and from *B. caccae* by being α-fucosidase negative.

(MIC, ≤8 μg/ml). Resistance was shown to penicillin G (MIC, ≥32 μg/ml), cefotetan (MIC, ≥256 μg/ml), and vancomycin (MIC ≥32 μg/ml) by all strains. All strains were β-lactamase positive.

To assess the genealogical affinity between the unknown bacteria and their relationship with other taxa, their 16S rRNA gene sequences were determined. Pairwise analysis showed that all of the isolates of the same group were phylogenetically closely related to each other (>99.5% sequence similarity). Sequence searches of GenBank and Ribosomal Database Project libraries revealed that the unknown organism was a member of the *Cytophaga-Flavobacter-Bacteroides* phylum, and it represents a previously unknown subline within the *Porphyromonas* subgroup (6). It has a high sequence similarity (99.2%) to altered Schaedler flora strain *Bacteroides* sp. ASF 519, and *B. merdae* was the phylogenetically closest valid species. A tree, constructed by the neighbor-joining method, depicting the phylogenetic affinity of the novel bacterium as exemplified by strain CCUG 48944^T, is shown in Fig. 1 and confirms the placement of the novel bacterium in the second subcluster of the *Porphyromonas* phylogenetic cluster. It is evident from the branching pattern in the tree that the novel species possesses a close relationship with *B. merdae* and *B. distasonis*, and bootstrap resampling showed this relationship to be statistically significant (99% recovery in 1,000 resamplings). Pairwise comparison revealed approximately 5.5% sequence divergence between the novel bacteria and the type strain of its closest valid species, *B. merdae*, based on almost the full length of the 16S rRNA gene sequences (1,400 nucleotides). Furthermore, a DNA-DNA reassociation value of 17.8% was observed between isolates WAL 12034^T and ATCC 43184^T (*B. merdae* type strain), thereby confirming that the unidentified bacterium represents a previously unknown subline within the second subcluster of the *Porphyromonas* phylogenetic cluster.

Support for the separation of the unknown bacterium from its related bacterial species also comes from the phenotypic characterization. The unknown organism can be readily distinguished from species of the genera *Tannerella*, *Prevotella*, and *Porphyromonas* by its resistance to 20% bile. In addition, the unknown bacterium is highly fermentative, in contrast to asaccharolytic species in the genus *Porphyromonas* and moderately saccharolytic species in *Prevotella*. It also can be readily distinguished from the genera *Dysgonomonas* and *Tannerella* by long-chain-fatty-acid analysis. The fatty-acid profile of the novel bacterium is incompatible with those of the species of the genera *Dysgonomonas* and *Tannerella*. *Dysgonomonas* has iso-$C_{14:0}$ as one of the major fatty acids, and *Tannerella* has a much higher ratio (>20) of anteiso-$C_{15:0}$ to iso-$C_{15:0}$ than that for the novel bacterium. Within the second subcluster of the *Porphyromonas* phylogenetic cluster, although 16S rRNA gene sequence analysis showed the closest phylogenetic relative to the unknown bacterium is *B. merdae*, it can be distinguished easily from *B. merdae* by several biochemical characteristics, such as by strongly producing β-glucosidase (tested with Rosco tablets) and α-glucosidase and strongly hydrolyzing esculin. In addition, by using RapID ANA II, the unknown bacterium showed a strongly positive reaction for phenylalanine aminopeptidase, whereas *B. merdae* was either negative or only weakly positive. In addition, the cellular fatty acid composition of the unknown was similar to that of *B. merdae* except that the ratio of 17:0 iso 3OH to 17:0 anteiso 3OH in the whole-cell methanolysates of the unknown species was lower than that of *B. merdae* (ratio ranges from 1.4 to 2.2 for the unknown bacterium and from 6.1 to 8.3 for *B. merdae*). The unknown bacterium can also be differentiated from *B. distasonis* by producing β-glucuronidase (tested by Rosco tablets), whereas *B. distasonis* does not. Furthermore, using RapID ANA II and API ZYM, β-glucosidase activity was not detected in the unknown bacterium, in contrast to *B. distasonis*. The characteristics for distinguishing the unknown bacteria from their phenotypically or phlogenetically closely related species are summarized in Table 2.

DISCUSSION

In this study, we report on the characterization of a group of strains of a novel bacterium that is isolated from clinical infections and is likely of intestinal origin. The strains were isolated from seven different patients. Four patients had appendicitis;

the strains were isolated from their appendix tissue. All the other three patients had peritonitis and abdominal abscess, and one of them had wound dehiscence and evisceration. Background factors were cecal inflammation of undetermined source, diverticulitis, and necrotic ileum in an incarcerated umbilical hernia. They are not commonly encountered in clinical specimens They were always found in mixed culture and were not recovered in blood cultures or in very serious infections. This group of clinical isolates was misidentified as *B. merdae* by routine biochemical tests. However, 16S rRNA sequencing revealed approximately 5.5% sequence divergence between the novel species and *B. merdae*. 16S rRNA sequence analysis showed that the novel bacterium has a close affinity with the second subcluster of the *Porphyromonas* cluster, which includes *B. merdae* and *B. distasonis*, with a mean sequence similarity of about 93.6%. It also displayed a lesser close phylogenetic relationship with the genera *Dysgonomonas* and *Tannerella* (with an average sequencing similarity of 88.5% and 90.5%, respectively). Other taxa displayed significantly lower levels of sequence similarity, including *Porphyromonas*, *Bacteroides sensu stricto*, *Prevotella*, and *Bacteroides splanchnicus*. It is evident from the described findings that the unidentified bacterium is not a species of *Bacteroides* sensu stricto affiliated with the type species (Fig. 1); it represents a hitherto-unknown line within the second subcluster of the *Porphyromonas* cluster, displaying a loose affinity (with an average similarity of 86%) with the first subcluster (the genus *Porphyromonas*). These data agree with previous findings (6); the deep branching position of the unknown bacterium suggested that a novel genus should be established to accommodate these three species. However, current phenotypic tests do not separate it from *Bacteroides* sensu stricto. Until the genera are revised along phylogenetic lines, the novel bacterium conforms to the present definition of the genus, and as such, this is the only appropriate home for the organism. Support for the separation of the unknown bacterium from related taxa also comes from phenotypic considerations. Table 2 summarizes the key characteristics for identification and differentiation of the novel bacterium that we are describing here from the other related taxa. Based on both phenotypic and genotypic evidence, it is clear that this group of unknown isolates recovered from infections of intestinal origin in humans represents a novel species.

We propose the unknown bacterium be classified as a new *Bacteroides* species, "*Bacteroides goldsteinii* sp. nov."

Description of "*Bacteroides goldsteinii* sp. nov." "*Bacteroides goldsteinii*" (in honor of the outstanding infectious disease clinician who has done a lot of work with anaerobes, Ellie C. Goldstein.). Cells are rod shaped, 0.9 to 1.5 μm by 1.2 to 10 μm. Colonies on Brucella blood agar plates at 48 h are gray, circular, convex, entire, and opaque and attain a diameter of 1 to 2 mm. Obligately anaerobic. Indole, urease, and nitrate negative. Esculin is hydrolyzed. Resistant to 20% bile. Acid is produced from cellobiose, glucose, rhamnose, sucrose, and xylose but not from arabinose, trehalose, and xylan. In peptone yeast broth and peptone yeast glucose broth, major amounts of acetic and succinic acids and minor amounts of isovaleric acid, propionic acid, and formic acid are produced by all isolates. Using the API ZYM, rapid ID 32A, and RapID ANA II systems, all isolates of the same group produced the same profile. Positive reactions were obtained for α-glucosidase, α-galactosidase, β-galactosidase, β-*N*-acetyl-glucosaminidase, naphthol-AS-Bl-phosphohydrolase, acid phosphatase, alkaline phosphatase, leucine arylamidase, *p*-nitrophenylphosphatase, arginine arylamidase, leucyl glycine arylamidase, leucine arylamidase, phenylalanine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, leucyl glycine aminopeptidase, glycine aminopeptidase, phenylalanine aminopeptidase, arginine aminopeptidase, and serine aminopeptidase. All the other tests were negative. α-Fucosidase was negative by all three systems. Mannose and raffinose were fermented when tested by the rapid ID 32A system. Using Rosco diagnostic tablets (Rosco, Taastrup, Denmark), β-xylosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase (*o*-nitrophenol-β-D-galactopyranoside), β-*N*-acetyl-glucosaminidase, alkaline phosphatase, and α-arabinosidase were detected; α-arabinosidase was tested as positive only by Rosco tablets. The principal long-chain cellular fatty acids of the isolates were anteiso-15:0 and iso-3-OH-17:0 (25 to 28% and 18 to 23% of the total, respectively). Significant amount of 18:1 *w*9*c* and anteiso-17:0 3OH (11 to 16% and 9 to 15% of the total, respectively) were also present. Agar dilution tests showed that the strains were susceptible to metronidazole (MIC, ≤2 μg/ml), and ertapenem (MIC, ≤1 μg/ml). Some resistance was seen with clindamycin (MIC, ≤8 μg/ml). Resistance was shown to penicillin G (MIC, ≥32 μg/ml), cefotetan (MIC, ≤256 μg/ml), and vancomycin (MIC, ≤32 μg/ml) by all strains. All strains were β-lactamase positive.

Isolated from human clinical specimens of intestinal origin. Habitat is probably the human gut. The type strain is WAL 12034^T = ATCC BAA-1180^T = CCUG 48944^T. The G+C content of the type strain is 43 mol%.

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